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(54) **Title:** METHODS FOR DIAGNOSING DISORDERS AND DISEASES ASSOCIATED WITH RYANODINE RECEPTORS AND METHODS FOR IDENTIFYING COMPOUNDS THAT AFFECT RYANODINE RECEPTORS

(57) **Abstract:** The present invention relates to methods for diagnosing disorders and diseases associated with ryanodine receptor function and/or associated with mutations in ryanodine receptors. The present invention also provides methods of indentifying compounds that affect ryanodine receptor function and that may be useful for treating disorders and diseases associated with abnormal or defective ryanodine receptor function, and various other methods. The methods may be performed using samples of peripheral blood, containing lymphocytes, and are therefore far less invasive than methods that involve obtaining samples tissue from other sources.

WO 2007/143112 A2

METHODS FOR DIAGNOSING DISORDERS AND DISEASES ASSOCIATED WITH RYANODINE RECEPTORS AND METHODS FOR IDENTIFYING COMPOUNDS THAT AFFECT RYANODINE RECEPTORS

[0001] This application claims the benefit of priority of International Application PCT/US2006/32405 filed August 17, 2006, and U.S. Application No: 60/810,748 filed June 2, 2006, and U.S. Application No: 60/904,348 filed February 28, 2007, and U.S. Application No: 60/866,556 filed on November 20, 2006. The disclosures of these applications are hereby incorporated by reference into this application in their entireties.

[0002] In one aspect the present invention relates to methods for diagnosing disorders and diseases associated with abnormal or defective ryanodine receptor function. These methods can be performed using a sample of lymphocytes and/or blood containing lymphocytes, from a subject, and are therefore far less invasive than methods that involve obtaining samples of cardiac muscle, skeletal muscle, or brain tissue, from subjects. In other aspect the invention provides methods of screening for compounds that affect ryanodine receptor function and that may be useful for treating disorders and diseases associated with abnormal or defective ryanodine receptor function. Such screening methods also utilize peripheral blood, containing lymphocytes. These and other embodiments of the invention are described herein.

BACKGROUND OF THE INVENTION

[0003] Intracellular calcium (Ca^{2+}) regulation plays an important role in animal physiology. Among the molecules that participate in controlling cystolic calcium levels are ryanodine receptors (RyRs), named for their affinity to the plant alkyloid ryanodine. RyRs are ligand gated calcium channels that regulate the release of calcium ions from intracellular storage sites, such as sarcoplasmic reticulum (SR), and are especially important in neurons and muscle cells. RyRs are stimulated by a positive feedback mechanism that is known as calcium-induced calcium release, with a rise in intracellular free Ca^{2+} concentration triggering the release of even more Ca^{2+}.

[0004] To date, three related isoforms of RyR are known, including RyRl, RyR2 and RyR3, which are expressed widely in animal tissues but in distinct sub-patterns. In striated muscle, for example, RyRl is preferentially expressed in skeletal muscle tissue, while RyR2 is preferentially expressed in cardiac tissue. In the brain, RyRl is preferentially expressed in Purkinje cells, RyR2 is expressed in cerebellum granule cells, and RyR3 is preferentially expressed in multiple areas, including the hippocampus, caudate/putamen, the olfactory

bulb, and olfactory tubercle. More generally, RyRs have been observed within cell bodies, axons, dendrites, spines and dendritic shafts.

[0005] RyRs comprise four channel subunits, or monomers, in association with multiple regulatory proteins. Homomeric RyRs are well known. Skeletal muscle RyR1, for example, comprises four RyR1 monomers. Cardiac muscle RyR2 comprises four RyR2 monomers. Heteromeric RyRs are also possible, however, as multiple receptor isoforms can be present in the same tissue. In addition to forming a channel, the RyR subunits form a regulatory region via their cytosol-projecting domains. This region provides a scaffold that can interact with many cytosolic modulators, including channel binding proteins, kinases, and phosphatases. Collectively, the RyR channel and associated proteins constitute a macromolecular channel complex, which is modulated by extracellular and intracellular signals, such as those triggering muscle contraction and nerve activity.

[0006] One important RyR modulator is calstabin (FK506-binding protein or FKBP), which encodes an approximately 12-kilodalton trans peptidyl-prolyl transferase. Two highly related calstabin isoforms, FKBP12 (calstabin-1) and FKBP12.6 (calstabin-2), are known to date. Calstabin binding to RyR is stoichiometric, i.e., one calstabin protein per RyR subunit. Under physiological conditions, calstabin-1 binds selectively to RyR1 and RyR3, and calstabin-2 binds predominantly with RyR2. In the heart, calstabin binding helps stabilize the RyR2 channel by decreasing its open probability, minimizing calcium leak into the cytoplasm and thereby preventing aberrant activation during the resting phase of the cardiac cycle. Calstabin binding also facilitates coupled gating between neighboring RyR2 channels, which is thought to be important for promoting efficient excitation-contraction in muscle.

[0007] Genetic studies support the view that calstabin binding to RyR plays a key physiological role in maintaining proper RyR function. Mice engineered for calstabin-2 haploinsufficiency show increased arrhythmia susceptibility and abnormal calcium physiology in the heart, including diastolic calcium release, monophasic action potential alternans, and bidirectional ventricular tachycardia (VT). Lehnart et al., *Proc, Natl Acad. Sci.* 2006;103;7906-7910. Calstabin-2 deficient cardiomycytes exhibit SR calcium leak-induced aberrant transient inward currents in diastole, consistent with DADs. In humans, the genetic disorder, catecholaminergic polymorphic ventricular tachycardia (CPVT), has been linked to RyR2 missense mutations, all of which decrease the binding affinity of calstabin-2 for RyR2. CPVT causes SCD in up to 50% of afflicted individuals by 35 years of age, CPVT

individuals have structurally normal hearts and do not develop ventricular arrhythmias when at rest, but are prone to ventricular arrhythmias in response to stress or exercise. Studies of heterologously expressed CPVT-mutant RyR2 channels reveal a significant intracellular leak during beta-adrenergic stimulation.

[0008] Similarly, skeletal muscle defects, which are among the major symptoms accompanying heart failure and include shortness of breath (resulting from diaphragmatic weakness) and intolerance to exercise (resulting from limb muscle fatigue), implicate aberrant regulation of RyR1, which is preferentially expressed in skeletal muscle tissue. Functional defects of RyR1 channels in HF skeletal muscle are analogous to those of RyR2 channels in HF myocardium, and include PKA-hyperphosphorylation of RyR and depletion of calstabin-1. Mutations in the RyR1 channel have also been linked to skeletal muscle diseases. For example, RyR1 mutations account for most cases of malignant hyperthermia (MH) and central core disease (CCD), which are autosomal dominant disorders. Under experimental conditions, MH mutant RyR1 channel has a phenotype similar to CPVT mutations in RyR2, including enhanced activity under resting conditions and Ca^{2+} leak from the SR to the cytoplasm.

[0009] Perturbed RyR signaling has also been implicated in neurodegenerative disorders. For example, the presence of RyR antibodies in blood serum has been associated with myasthenia gravis, the most common primary disorder of neuromuscular transmission. Notably, patients with myasthenia gravis come to the physician complaining of specific muscle weakness and not of generalized fatigue. RyR dysfunction is also associated with Alzheimer's disease (AD). Elevated RyR3 levels are observed in brain tissues isolated from genetically modified mice that express mutant Presenilin 1 at normal levels. Similarly, levels of RyR3 are greatly increased in PC 12 cells overexpressing mutant human Presenilin 1. More generally, exaggerated intracellular Ca^{2+} levels have been associated with expression of AD mutations in young mice. These disruptions persist throughout the animal's lifetime, indicating that they are not merely an acceleration of normal aging, but are instead a discrete "calciumopathy" related to a modulation of RyR signaling associated with PS 1 mutations.

[0010] These observations indicate that RyR-mediated calcium signaling is subject to tight regulation in neurons, and that disrupting this regulation can lead to disease. Thus, intracellular calcium signaling is critical to neuronal signaling, with the main source of

intracellular calcium being released from internal stores and RyRs, but not inositol 1,4,5-triphosphate (IP3) receptors, which are another endoplasmic calcium release channel, appearing to modulate cellular protein synthesis. Alcazar *et al., J. Neurochem.* 1997;69:1703-1708.

[0011] These studies underscore the need for methods of diagnosing diseases and disorders associated with altered RyR function, and methods for identifying substances that modulate the binding between calstabin and RyRs and that can be used to treat RyR-related disorders. The present invention addresses these needs.

SUMMARY OF THE INVENTION

[0012] In one aspect the present invention relates to methods for diagnosing disorders and diseases associated with abnormal or defective ryanodine receptor function. The methods of the invention can be performed using a sample of blood , including but not limited to peripheral blood, white blood cells or lymphocytes, from a subject, and are therefore far less invasive than methods that involve obtaining samples of cardiac muscle, skeletal muscle, or brain tissue, from subjects. In other aspect the invention provides methods of screening for compounds that affect ryanodine receptor function and that may be useful for treating disorders and diseases associated with abnormal or defective ryanodine receptor function. Such screening methods also utilize blood samples such as blood containing lymphocytes. Other aspects of the present invention include methods of monitoring disease progression by monitoring the composition and characteristics of the RyR protein complex, for example but not limited to the level of ryanodine phosphorylation in blood samples (e.g., peripheral blood, white blood cells or lymphocytes) from a subject that has or is at risk of developing a disease or condition associated with the ryanodine receptor. Other aspects of the present invention include methods of monitoring disease progression by quantifying the amount of calstabin bound to a ryanodine receptor in blood samples (e.g., peripheral blood, white blood cells or lymphocytes) from a subject that has or is at risk of developing a disease or condition associated with the ryanodine receptor. Other aspects of the invention include methods of monitoring drug efficacy by quantifying the amount of calstabin bound to a ryanodine receptor in blood samples (e.g., peripheral blood, white blood cells or lymphocytes) from a subject before, during and after treatment with a test drug. These and other embodiments of the invention are described herein.

[0013] The present invention relates, in certain aspects, to methods for diagnosing disorders and diseases associated with abnormal or defective ryanodine receptor function that can be performed using a sample of lymphocytes from a subject, or a samples of peripheral blood containing lymphocytes from a subject, and are therefore far less invasive than methods that involve obtaining samples of cardiac muscle, skeletal muscle, or brain tissue, from subjects. In other embodiments the invention provides methods of screening for compounds that affect ryanodine receptor function and that may be useful for treating disorders and diseases associated with abnormal or defective ryanodine receptor function. Such screening methods also utilize lymphocytes obtained from a subject, or a samples of blood containing lymphocytes obtained from a subject. These and other embodiments of the invention are described herein.

[0014] In one embodiment, the present invention provides methods of diagnosing diseases or disorders associated with abnormal ryanodine receptors or abnormal ryanodine receptor function in subject by obtaining a sample of blood from the subject (e.g. peripheral blood), or a sample of white blood cells, (e.g. lymphocytes) derived from a blood sample from the subject, and testing the ryanodine receptor in that sample to determine if the ryanodine receptor is abnormal such that it may be associated a some disease or disorder.

[0015] Sources of lymphocytes or blood or blood cells that can be used in the context of the present invention include, but are not limited to, peripheral blood, bone marrow, lymph nodes, spleen, thymus, as well as all cell types that are obtainable from a peripheral blood sample. Examples include, but are not limited to, all lineages of mature and immature, normal and abnormal, functional and dysfunctional leukocytes, lymphocytes, T-lymphocytes (T-cells), B-lymphocytes (B-cells), white blood cells, osteoclasts, tumor cells, natural killer cells, leukocytes, granulocytes, peripheral blood mononuclear cells (PBMC), plasma, thrombocytes, basophils, macrophages and dendritic cells. For each of the methods described herein, any of the above listed sources of cells can be used. In preferred embodiments, the methods of the invention utilize lymphocytes, and preferably the lymphocytes are in or from a source that can be obtained from a subject in a minimally invasive manner, such as peripheral blood, or a fraction obtained from peripheral blood.

[0016] For example, in one embodiment, the present invention provides a method of diagnosing a disease or disorder associated with abnormal ryanodine receptor function in a test subject comprising: (a) obtaining a blood sample from a test subject, (b) determining the

amount of a calstabin protein bound to a ryanodine receptor protein in the blood sample from the test subject, and (c) comparing the amount of the calstabin protein bound to the ryanodine receptor in the test subject to a control measurement for the amount of the calstabin protein bound to the ryanodine receptor, whereby a lower amount of calstabin protein bound to the ryanodine receptor in the test subject as compared to the control measurement indicates that the test subject may have a disease or disorder associated with abnormal ryanodine receptor function. The control measurement may be determined by any suitable method, for example by: (a) obtaining a tissue sample containing a ryanodine receptor and a calstabin protein from a control subject known to have normal ryanodine receptor function, and (b) determining the amount of the calstabin protein bound to the ryanodine receptor protein in the tissue sample from the control subject, thereby giving a control measurement for the amount of the calstabin protein bound to the ryanodine receptor in a subject known to have normal ryanodine receptor function. The control measurement may also be obtained from a prior measurement of the amount of calstabin protein bound to ryanodine receptor obtained using a ryanodine receptor known to have normal function and/or structure and/or normal interaction with calstabin.

[0017] In another embodiment, the present invention provides a method of diagnosing a disease or disorder associated with abnormal ryanodine receptor function in a test subject comprising: (a) obtaining a blood sample from a test subject, (b) obtaining a blood sample from a control subject known to have normal ryanodine receptor function, (c) determining the amount of a calstabin protein bound to a ryanodine receptor protein in the peripheral blood sample from the test subject, (d) determining the amount of the calstabin protein bound to the ryanodine receptor protein in the peripheral blood sample from the test subject, and (e) comparing the amount of the calstabin protein bound to the ryanodine receptor in the test subject to the amount of the calstabin protein bound to the ryanodine receptor in the control subject, whereby a lower amount of calstabin protein bound to the ryanodine receptor in the test subject as compared to the control subject indicates that the test subject may have a disease or disorder associated with abnormal ryanodine receptor function.

[0018] In the above embodiment, and indeed in any and all embodiments described herein that involve determining the amount of a calstabin protein that is bound to a ryanodine receptor protein, any suitable method known in the art for detecting and quantifying the interaction between two proteins may be used. For example, in one embodiment,

determining the amount of a calstabin protein that is bound to a ryanodine receptor protein may be performed by immunoprecipating the ryanodine receptor protein and detecting, and quantifying the amount of, the calstabin protein present in the immunoprecipated ryanodine receptor protein complex, or by immunoprecipating the calstabin protein and detecting, and quantifying the amount of, the ryanodine receptor protein present in the immunoprecipated calstabin protein complex. When such methods are used, the ryanodine receptor protein complex may be immunoprecipated using an anti-ryanodine receptor antibody, and/or the calstabin protein may be immunoprecipated using an anti-calstabin antibody. To detect the presence of calstabin in an immunoprecipated ryanodine receptor protein complex, an anti-calstabin antibody may be used. Conversely, to detect the presence of a ryanodine receptor in an immunoprecipated calstabin protein complex, an anti-ryanodine receptor antibody may be used. Methods of detecting proteins using antibodies are well known in the art, and any such method can be used. In certain embodiments, instead of, or in addition to using antibody-based detection, the ryanodine receptor may be radiolabeled, and the amount of ryanodine receptor bound to calstabin is determined by detecting and quantifying the radiolabel. Similarly, the calstabin protein may be radiolabeled, and the amount of ryanodine receptor bound to calstabin may be determined by detecting and quantifying the radiolabel in the calstabin protein. In certain other embodiments, instead of, or in addition to using antibody-based detection, the ryanodine receptor may be fluorescently labeled, and the amount of ryanodine receptor bound to calstabin may be determined by detecting and quantifying the fluorescent label. Similarly, the calstabin protein may be fluorescently labelled, and the amount of ryanodine receptor bound to calstabin may be determined by detecting and quantifying the fluorescent label.

[0019] In another embodiment, the present invention provides methods for detecting mutations in ryanodine receptors, such as RyR1, RyR2, and RyR3 in peripheral blood samples, containing lymphocytes, obtained from a subject. Even if the ultimate effect of the RyR receptor mutation is primarily in a tissue such as heart, muscle, or the brain, the presence of a mutation in a RyR gene, or in a RyR cDNA, can be detected in a peripheral blood sample, thereby provididing an easier and less invasive method of detecting a RyR mutation than corresponding methods that use biopsy material from cardiac, muscle or brain tissue.

[0020] Thus, in one embodiment the present invention provides a method for detecting a mutation in a ryanodine receptor, comprising (a) obtaining a blood sample from the subject, (b) obtaining a DNA sample from the blood sample from the test subject, (c) determining the nucleotide sequence of the DNA encoding a ryanodine receptor in the DNA sample from the test subject, (d) obtaining a blood sample from a control subject subject known to have no mutations in a ryanodine receptor, (e) obtaining a DNA sample from the l blood sample from the control subject, (f) determining the nucleotide sequence of the DNA encoding the ryanodine receptor in the DNA sample from the control subject, and (g) comparing the nucleotide sequence of the ryanodine receptor in the test subject and the control subject, whereby a difference between the nucleotide sequence of the DNA encoding the ryanodine receptor in the test subject and the control subject, indicates that the test subject has a mutation in the gene encoding the ryanodine receptor.

[0021] In another embodiment, the present invention provides a method for detecting a mutation in a ryanodine receptor in a test subject, comprising: (a) obtaining a blood sample from the subject, (b) obtaining a DNA sample from the blood sample from the test subject, (c) determining the nucleotide sequence of the DNA encoding a ryanodine receptor in the DNA sample from the test subject, and (d) comparing the nucleotide sequence of the ryanodine receptor in the test subject to the nucleotide sequence of the corresponding wild type ryanodine receptor, whereby a difference between the nucleotide sequence of the DNA encoding the ryanodine receptor in the test subject and the nucleotide sequence of the corresponding wild type ryanodine receptor, indicates that the test subject has a mutation in the gene encoding the ryanodine receptor.

[0022] The methods for detecting mutations provided herein can be used to detect mutations in genomic DNA or in samples of cDNA, such as cDNA generated by reverse transcription of mRNA obtained from the lymphocytes. The mutations can be detected using any suitable method known in the art, such as standard nucleotide sequencing methods, PCR-based methods, restriction fragment length polymorphism analysis, and the like.

[0023] In certain embodiments, the nucleotide sequence of the wild type ryanodine receptor may be obtained from a public nucleotide sequence database, such as the Genbank database or the human genome database. The nucleotide sequence of the wild type ryanodine receptor may be also obtained from publications in which the wild-type nucleotide sequence of a

RyR is provided, or may also be obtained by sequencing a RyR gene of cDNA from an individual known to have a wild type version of that RyR gene.

[0024] In certain embodiments, it may also be desirable to compare the sequence of the RyR gene in the test subject with that of an individual known to have a specific mutation in one of their RyR genes.

[0025] In another embodiment, the present invention provides amethod of determining the amount of ryanodine receptor phosphorylation in a test subject comprising: (a) obtaining a sample of blood from a test subject, (b) determining the amount of a phosphorylation of a ryanodine receptor protein in the blood sample, (c) comparing the amount of phosphorylation of the ryanodine receptor in the test subject to a control measurement of the amount of phosphorylation of a ryanodine receptor, whereby a higher amount of the ryanodine receptor phosphorylation in the test subject as compared to the control measurement indicates that the test subject has or is at risk of developing a ryanodine receptor hyperphosphorylation defect and has or is at risk of developing, a disease or disorder associated with ryanodine receptor hyperphosphorylation. The control measurement may be made using any suitable method known in the art, for example the control measurement may be determined by: (a) obtaining a tissue sample containing a ryanodine receptor from a control subject known to have normal ryanodine receptor phosphorylation, and (b) determining the amount of phosphorylation of the ryanodine receptor protein in the tissue sample from the control subject, thereby giving a control measurement for the amount of phosphorylation of the ryanodine receptor in a subject known to have normal ryanodine receptor phosphorylation. The control measurement may also be obtained from a prior measurement of the amount of phosphorylation of the ryanodine receptor obtained using a ryanodine receptor known to have normal phosphorylation, such as normal PKA phosphorylation.

[0026] In another emnodiment, the present invention provides a method of determining the level of ryanodine receptor phosphorylation in a test subject comprising: (a) obtaining a blood sample from a test subject, (b) obtaining a blood sample from a control subject known to have normal ryanodine receptor phosphorylation, (c) determining the amount of a phosphorylation of a ryanodine receptor protein in the blood sample from the test subject, (d) determining the amount of phosphorylation of the ryanodine receptor protein in the blood sample from the control subject, and (e) comparing the amount of phosphorylation of the

ryanodine receptor in the test subject to the amount of phosphorylation of the ryanodine receptor in the control subject, whereby a higher amount of the ryanodine receptor phosphorylation in the test subject as compared to the control subject indicates that the test subject may have a ryanodine receptor hyperphosphorylation defect and may have, or be at risk of developing, a disease or disorder associated with ryanodine receptor hyperphosphorylation.

[0027] Methods of detecting and quantifying phosphorylation of proteins, and RyRs specifically, such as PKA phosphorylation, are known in the art, and any such method can be used. For example, radioactive inorganic phosphorous isotopes (such as 32P or 33P) can be used to tag cellular phosphorylated proteins, or antibodies that specifically bind to phosphorylated proteins can be used.

[0028] In yet another embodiment, the present invention provides a method for identifying, or screening for, an agent, such as a chemical compound, that enhances binding of a calstabin protein to a ryanodine receptor protein, or inhibits dissociation of a calstabin protein from a ryanodine receptor protein, comprising (a) obtaining a blood sample from a subject, (b) contacting a first portion of the blood sample with an agent that causes dissociation of a calstabin protein from a ryanodine receptor protein, (c) measuring the amount of a calstabin protein bound to a ryanodine receptor protein in the first portion of the blood sample, (d) contacting a second portion of the blood sample with an agent that causes dissociation of a calstabin protein from a ryanodine receptor protein and a test compound, (e) measuring the amount of the calstabin protein bound to the ryanodine receptor protein in the second portion of the blood sample, and (f) comparing the amount of the calstabin protein bound to the ryanodine receptor protein in the first portion of the blood sample or the second portion of the blood sample, whereby a higher amount of calstabin protein bound to the ryanodine receptor protein in the second portion of the blood sample as compared to the first portion of the blood sample indicates that the test compound either enhances binding of the calstabin protein to the ryanodine receptor protein, or inhibits dissociation of the calstabin protein from the ryanodine receptor protein.

[0029] In another embodiment, the present invention provides an *in vivo* method for identifying a chemical compound that enhances binding of a calstabin protein to a ryanodine receptor protein, or inhibits dissociation of a calstabin protein from a ryanodine receptor protein, comprising: (a) administering to an animal a physiologically effective amount of a

regulating compound that decreases binding of a calstabin protein to a ryanodine receptor protein, or causes dissociation of a calstabin protein from a ryanodine receptor protein; (b) obtaining a first blood sample from the animal, (c) subsequently administering to the animal a physiologically effective amount of a regulating compound that decreases binding of a calstabin protein to a ryanodine receptor protein, or causes dissociation of a calstabin protein from a ryanodine receptor protein, and test compound, (d) obtaining a second blood sample from the animal, (e) measuring the amount of a calstabin protein bound to a ryanodine receptor protein in the first sample and the second sample, (f) comparing the amount of the calstabin protein bound to the ryanodine receptor protein in the first sample to the amount of the calstabin protein bound to the ryanodine receptor protein in the second sample, whereby a higher amount of calstabin protein bound to ryanodine receptor protein in the second sample as compared to the first sample indicates that the test compound enhances binding of the calstabin protein to the ryanodine receptor protein, or inhibits dissociation of the calstabin protein from the ryanodine receptor protein.

[0030] In another embodiment, the present invention provides an *in vivo* method for identifying a compound that produces a change in calstabin binding to a ryanodine receptor, which method comprises: (a) administering to a first animal a physiologically effective amount of a regulating compound that regulates calstabin binding to a ryanodine receptor; (b) obtaining a first blood sample from the first animal, (c) measuring the amount of calstabin bound to the ryanodine receptor in the first blood sample; (d) administering to a second animal, which in one embodiment may be substantially genetically identical to the first animal, the same relative to weight amount of the regulating compound in combination with an amount of a test compound,(e) obtaining a second sample of blood from the second animal, (f) measuring the amount of calstabin bound to the ryanodine receptor in the second blood sample; and (g) comparing the measured amounts of calstabin bound to the ryanodine receptor in the first and the second blood samples to determine whether the test compound changes the amount of calstabin bound to the ryanodine receptor, wherein an increased amount of calstabin bound to the ryanodine receptor in the second lymphocyte sample is indicative of a test compound which increases the binding between calstabin and the ryanodine receptor or inhibits the dissociation of calstabin from the ryanodine receptor.

[0031] In the above *in vivo* methods, the regulating compound may be a beta-adrenergic agonist. In a preferred embodiment, the beta-adrenergic agonist is isoproterenol or "ISO", as

is used in Example 1. In other embodiments, the regulating compound may be an agonist of the cAMP pathway.

[0032] The above screening methods, and variations thereof, can be used to identify any class of agent that has the desired effect on a RyR protein or the RyR:calstabin interaction. In one preferred embodiment, the agent is a 1,4, benzothiazepine compound, such as a 1,4, benzothiazepine compound that may be described by chemical Formula I described in the below detailed description. Example 1 and Figure 1 show that such an assay can be used to demonstrate the efficacy of compounds ARM036 and ARM 064 on the RyR:calstabin interaction in lymphocytes obtained from a blood sample from rats. These two compounds are 1,4, benzothiazepine compounds. However, the assay can also be used to dentify any class of agent that has the desired effect on a RyR protein or the RyR:calstabin interaction.

[0033] In the above *in vivo* methods, the compounds may be administered by any suitable route, such as orally, subcutaneously, or via an implanted osmotic pump. In preferred embodiments, the regulating compound is administered subcutaneously via an osmotic pump for at least about 3 days, or for at least about about 5 days.

[0034] In another embodiment, the present invention provides a method of diagnosing a disease or disorder associated with leak of calcium through a ryanodine receptor in a test subject comprising: (a) obtaining a blood sample, containing lymphocytes, from a test subject, (b) obtaining a blood sample, containing lymphocytes, from a control subject known to have normal ryanodine receptor function, and (c) determining whether there is any calcium leak through the ryanodine receptor in the a blood samples obtained in the test subject and the control subject, whereby a higher level of calcium leak in the ryanodine receptor from the test subject as compared to the control subject, indicates that the test subject may have a disease or disorder associated with the leak of calcium through a ryanodine receptor. Un such methods, the step of determining whether there is any calcium leak through the ryanodine receptor is performed using an electrophysiological recording technique, such as single channel patch clamp recording an the like. Alternatively, calcium leak can be measured using a fluorescent intracellular calcium indicator or any other means known in the art for monitoring calcium flow through ion channels.

[0035] In each of the above embodiments, the blood sample can contain lymphocytes. In some embodiments, a sample of peripheral blood may be used. In some embodiments, a

sample of bone marrow cells may be used. In some embodiments, a sample of lymphocytes may be used. A buffy coat preparation may be produced from a blood sample using any method known in the art, such as the method described in the accompanying Example. The blood/lymphocyte sample may comprise B cells, T cells, natural killer cells, or any combination thereof, or any other type of lymphocyte known in the art that expresses at least one ryanodine receptor.

[0036] In each of the above embodiments, the calstabin protein may be calstabin 1 or calstabin 2, and the ryanodine receptor protein may be RyR1, RyR2, or RyR3, or any combination thereof.

[0037] In each of the above embodiments, the test subject, and the control subject if one is used, may be any animal for which it is desired to detect or diagnose a disease or disorder, or detect a mutation, or in which it is desired to screen the efficacy of certain compounds. In one embodiment, the subjects are mammals. In one preferred embodiment, the subjects are humans. In other preferred embodiments, such as drug screening methods, the preferred subjects may be rodents such as mice. The control subject and the test subject are preferably from the same species.

[0038] In each of the above embodiments, the diseases and disorders referred to may be any disease or disorder associated with altered function or activity of a ryanodine receptor, or associated with a mutation in a ryanodine receptor, or associated with an altered interaction between a ryanodine receptor and a calstabin protein. Examples of such diseases and disorders include, but are not limited to, cardiac disorders and diseases, skeletal muscular disorders and diseases, cognitive disorders and diseases, malignant hyperthermia, diabetes, and sudden infant death syndrome. Cardiac disorders and diseases include, but are not limited to irregular heartbeat disorders and diseases, exercise-induced irregular heartbeat disorders and diseases, sudden cardiac death, exercise-induced sudden cardiac death, congestive heart failure, chronic obstructive pulmonary disease, and high blood pressure. Irregular heartbeat disorders and diseases include, but are not limited to, atrial and ventricular arrhythmias, atrial and ventricular fibrillation, atrial and ventricular tachyarrhythmias, atrial and ventricular tachycardias, catecholaminergic polymorphic ventricular tachycardia (CPVT), and exercise-induced variants thereof. Skeletal muscular disorders and diseases include, but are not limited to, skeletal muscle fatigue, exercise-induced skeletal muscle fatigue, myopathies, muscular dystrophy, certain types of skelelal

muscle bladder disorders, and incontinence associated with skeletal muscle function. Cognitive disorders and diseases include, but are not limited to, Alzheimer's Disease, memory disorders, and age-dependent memory disorders.

[0039] In another embodiment, the present invention provides a method of assessing the efficacy of drug treatment, by performing one of the assays described above, such as those used to detect the RyR:calstabin interaction at various points in time before, during, and after drug therapy, thereby monitoring the efficacy of the drug therapy.

[0040] In another embodiment, the present invention provides a method of monitoring disease progression, by performing one of the assays described above, such as those used to detect the RyR:calstabin interaction or those used to evaluate the level of ryanodine phosphorylation, at various points before or following diagnosis of the disease, thereby monitoring the progression of the disease.

[0041] In another embodiment, the present invention provides an *in vivo* method for identifying a compound that produces a change in calstabin binding to a ryanodine receptor, which method comprises administering a test drug to an animal known to have hyperphosphorylated ryanodine; obtaining a sample of blood cells from the animal; measuring calstabin binding to ryanodine receptor in said animal; and comparing the amount of calstabin bound to ryanodine in the animal to the amount of calstabin bound to ryanodine in an untreated control animal known to have hyperphosphorylated RyR to determine whether the test compound changes calstabin binding to such ryanodine receptor.

BRIEF DESCRIPTION OF THE DRAWINGS

[0042] The above features and other advantages of the invention will become better understood by reference to the following detailed description, the Examples, and the accompanying Figure.

[0043] Figure 1 demonstrates provides data from an *in vivo* rat assay in which isoproterenol was used to destabilize binding of calstabin to RyRs, and then two test compounds, namely the 1,4, benzothiazpeines S36 and S64 were tested to see if they increased binding of calstabin to the RyRs. Calstabin binding was measured by immunoprecipitation of RyR from on buffy coat samples prepared from peripheral blood, and blotting using calstabin antibodies.

DETAILED DESCRIPTION

[0044] For a description of the major embodiments of the invention, please refer to the Summary of the Invention section above, and also the examples and claims provided herein. The below detailed description provides certain additional information to further describe information described in the Summary of the Invention, the examples and the claims.

[0045] Definitions

[0046] As used herein, the singular forms "a," "an," and "the" include plural references, unless the content clearly dictates otherwise. Thus, for example, reference to "a compound" includes a plurality of such compounds and equivalents thereof known to those skilled in the art, and reference to "the FKBP12.6 polypeptide" (also known as calstabin-2) is a reference to one or more FKBP12.6 polypeptides and equivalents thereof known to those skilled in the art, and so forth.

[0047] As used herein, the term "about" or "approximately" means within an acceptable range for the particular value as determined by one of ordinary skill in the art, which will depend in part on how the value is measured or determined, *e.g.*, the limitations of the measurement system. For example, "about" can mean a range of up to 20%, up to 10%, up to 5%, and up to 1% of a given value. Alternatively, particularly with respect to biological systems or processes, the term can mean within an order of magnitude, within 5-fold, within 2-fold, of a value.

[0048] As used herein, the term "treating" or "treatment" of a disorder or disease includes (1) preventing or delaying the appearance of clinical symptoms of the disorder or disease developing in a mammal that may be afflicted with or predisposed to the disorder or disease but does not yet experience or display clinical or subclinical symptoms of the disorder or disease; or (2) inhibiting the disorder or disease, i.e., arresting, reducing or delaying the development of the disease or a relapse thereof (in case of maintenance treatment) or at least one clinical or sub-clinical symptom thereof; or (3) relieving the disease or disorder, i.e., causing regression of the disease or disorder or at least one of its clinical or sub-clinical symptoms. The benefit to a subject to be treated is either statistically significant or perceptible to the patient or to the physician.

[0049] As used herein, the term "patient" or "subject" or "animal" refers to all members of the animal kingdom, particularly vertebrates, and especially mammals e.g., mice, rats, pigs, cats, and dogs. In the context of therapeutic and diagnostic applications, the term also includes humans.

[0050] As used herein, the terms diseases or disorders or conditions associated with a ryanodine receptor" includes disorders and diseases that can be treated by modulating the RyR receptors that regulate calcium channel functioning in cells. These disorders, diseases and conditions include, without limitation, cardiac disorders and diseases, skeletal muscular disorders and diseases, cognitive disorders and diseases, malignant hypethermia, diabetes, sudden infant death syndrome, and muscle fatigue (e.g., exercise-induced muscle fatigue, or muscle fatigue induced by a disease or disorder).

[0051] Examples of cardiac disorders and diseases include, but are not limited to, irregular heartbeat disorders and diseases; exercise-induced irregular heartbeat disorders and diseases; congestive heart failure; chronic obstructive pulmonary disease; and high blood pressure. Examples of irregular heartbeat disorders and diseases and exercise-induced irregular heartbeat disorders and diseases include, but are not limited to, atrial and ventricular arrhythmia; atrial and ventricular fibrillation; atrial and ventricular tachyarrhythmia; atrial and ventricular tachycardia; catecholaminergic polymorphic ventricular tachycardia (CPVT); and exercise-induced variants thereof.

[0052] Examples of skeletal muscular disorder and diseases include, but are not limited to, muscle fatigue that results from pathologies, illnesses, diseases, disorders or conditions that are associated with the RyR receptors that regulate calcium channel functioning in cells. Examples of such disorders and conditions include, but are not limited to, cardiac disease or disorder, defective skeletal muscle function, HIV Infection, AIDS, muscular dystrophy, cancer, malnutrition, exercise-induced muscle fatigue, age-associated muscle fatigue, renal disease, renal failure, bladder disorders, incontinence, malignant hypothermia, and central core disease.

[0053] Examples of cognitive disorders and diseases include, but are not limited to, forms of memory loss, age-dependent memory loss, and neurodegenerative disorders, including Alzheimer's Disease and myasthenia gravis.

[0054] As used herein, the term "inhibiting dissociation" includes blocking, decreasing, inhibiting, limiting or preventing the physical dissociation or separation of an FKBP subunit (or calstabin protein) from a RyR molecule (e.g., phosphorylated RyR molecule), and blocking, decreasing, inhibiting, limiting or preventing the physical dissociation or separation of the RyR molecule from an FKBP subunit (or calstabin protein).

[0055] As used herein, the term "increasing binding" or "enhancing binding" or "enhancing reassociation" includes enhancing, increasing, or improving the ability of RyR (e.g., phosphorylated RyR) to associate physically with FKBP/calstabin *(e.g.,* binding of approximately two fold or, approximately five fold, above the background binding of a negative control) in a tissue sample or cells of the subject and enhancing, increasing or improving the ability of FKBP/calstabin to associate physically with RyR *(e.g.,* binding of approximately two fold, or, approximately five fold, above the background binding of a negative control) in cells of the subject.

[0056] As used herein, the term "RyR" refers to a ryanodine receptor and includes RyRl, RyR2, and RyR3 subtypes of the ryanodine receptor. It also includes an "RyR protein" and an "RyR analog." An "RyR analog" is a functional variant of the RyR protein, having RyR biological activity that has about 60%, 70%, 80%, 85%, 90%, 95%, 97% or greater amino-acid-sequence homology with the RyR protein.

[0057] As used herein, the term "calstabin" includes calstabin-1 (FKBP 12) or calstabin-2 (FKBP 12.6). It also includes a "calstabin protein," "calstabin polypeptide," and "calstabin analog." A "calstabin analog" is a functional variant of the calstabin protein, having calstabin biological activity, e.g., ability to bind RyR that has about 60%, 70%, 80%, 85%, 90%, 95%, 97% or greater amino-acid-sequence homology with the calstabin protein. "RyR-bound calstabin" therefore includes RyRl-bound calstabin-1 (FKBP12), RyR2-bound calstabin-2 (FKBP12), and RyR3-bound calstabin-1 (FKBP12).

[0058] The terms "regulatory compound" and "regulating compound" have the same meaning. The term "regulatory compound" refers to a compound which modulates the interaction between calstabin and ryanodine.

[0059] As used herein, the term "modulate" includes decreasing, increasing, inhibiting, enhancing, stabilizing, and/or restabilizing an effect. For example, a regulating compound, such as a beta agonist, can have a regulatory effect on RyR2 in cardiac tissue by increasing

RyR phosphorylation by PKA, leading to decreased binding by calstabin-2. Accordingly, a test compound can modulate this effect by inhibiting the regulating compound-induced dissociation of calstabin and RyR; and/or by increasing binding between calstabin and RyR following such regulating compound-induced dissociation; stabilizing the RyR-calstabin complex, and even restabilizing the RyR complex by promoting the reassociation of calstabin to RyR.

[0060] Calstabin binding to RyR can be regulated by covalent modifications to RyR. For example, calstabin-2 binding to RyR2 is regulated by PKA phosphorylation of Ser2809 in RyR2. PKA phosphorylation of RyR2 decreases the binding affinity of calstabin-2, causing calstabin-2 dissociation and increasing RyR2 open probability (Po) and its sensitivity to Ca^{2+}-dependent activation. More generally, PKA-phosphorylated RyR is an important part of the "fight or flight" response, by which the sympathetic nervous system (SNS), in response to stress or exercise, enhances myocardial contractility and increases cardiac output. In particular, the activated sympathetic nervous system produces catecholamines (noradrenalin and adrenalin), which stimulate a beta adrenergic signaling cascade that results in a transient increase in PKA-phosphorylation of RyR2 at S2809. By decreasing the binding affinity of RyR2 for calstabin-2, this modification alters the biophysical properties of the channel such that there is an increased SR calcium release for a given calcium trigger.

[0061] Aberrations in intracellular calcium signaling are associated with a spectrum of diseases, including disorders of the heart, muscle, and brain. Disruptions in RyR function and its association with calstabin are implicated in at least some of these diseases.

[0062] The growing recognition that defective RyR regulation plays an important role in pathophysiological processes has prompted the view that normalizing RyR channel function under these conditions offers a valuable therapeutic approach to treat RyR-related disorders. For example, therapeutics that increase calstabin-2 binding to PKA-phosphorylated RyR2 may repair the diastolic Ca^{2+} leak in HF patients, halting disease progression, preventing deadly cardiac arrhythmias, and improving cardiac contractility. This is supported by the finding that mutant forms of calstabin having a negative charge that allows binding to PKA-phosphorylated RyR2 can rescue CPVT-linked RyR2 mutants that deplete the calstabin-2 channel complex *in vitro* and *in vivo*. Further, these calstabin mutants can rescue defective channel function in PKA-hyperphosporylated RyR2, and they complement defective channel function in a mutant RyR2 (S2808D) that mimics constitutive PKA phosphorylation. Further

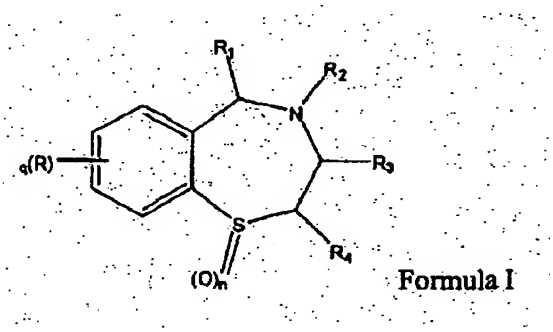
support is provided by recent studies involving the compound JTV519, a 1,4-benzothiazepine derivative that enhances the binding affinity of calstabin-2 for RyR2 even when the channel is PKA hyperphosphorylated. In a canine HF model, JTV519 slowed HF progression by inhibiting RyR2 hyperactivity and intracellular calcium leak. Masafumi et al., *Circulation.* 2003; 107:477-484. Similarly, in a mouse HF model, JTV519 increased calstabin-1 binding to RyR1 and improved skeletal muscle function. *Wehrens* et al., *Proc. Natl. Acad. Sci.* 2005;102:9607-9612; U.S. Pat. No. 6,489,125. In calstabin-2 haploinsufficient mice, JTV519 prevented diastolic Ca^{2+} leak and catecholamine-induced ventricular arrhythmias by increasing calstabin-2 binding to PKA-phosphorylated RyR2.

[0063] *In vivo* model of stimulation of hyperadrenergic state

[0064] In one aspect, the present invention is directed to the use of an *in vivo* animal model, for example a mouse or rat model, which mimics the effect of chronic sympathetic stimulation of ryanodine receptors in the heart or skeletal muscles, including phosphorylation by protein kinase A and dissociation of calstabin subunits. Chronically administered beta-adrenergic agonists, for example but not limited to isoproterenol ("ISO), by osmotic minipump, or any other method for *in vivo* delivery of a drug (e.g. IV and IP injection), mimics chronic sympathetic nervous system activation as occurring in disease processes in the heart, skeletal muscle, or other organs. In certain embodiments, chronic administration of isoproterenol to an animal, such as a rat or mouse, by osmotic pump infusion implanted subcutaneously for one week depletes calstabins from RyR. This depletion can be detected in peripheral blood samples, and buffy coat preparations made therefrom, thereby providing a non-invasive animal model by which this effect can be studied, and by which compounds that may increase binding of calstabin to RyRs can be screened for or studied. Such compounds are useful for the treatment of disorders and conditions associated with disruption of the RyR:calstabin interaction.

[0065] Chronically administering beta-adrenergic agonist for at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 days, to an animal leads to increased RyR phosphorylation, and decreased calstabin binding. In certain embodiments the agonist is isoproterenol. The effects of continuous or chronic administration of beta-adrenoreceptor agonist can be examined and quantified by isolating peripheral blood, containing lymphocytes, from the animal and measuring the levels of calstabin bound to RyR complexes. The effects of drug treatment on the levels of calstabin bound to RyR complexes can also be studied, in order to identify new

agents that may be useful for treatment of diseases and disorders affecting RyR function. Any agent that could potentially have an on the levels of calstabin bound to RyR can be studied using this assay. However, in a preferred embodiment, the method can be used to identify a therapeutically useful 1,4-benzothiazepine derivative as represented by Formula I:



Formula I

wherein

n is 0, 1, or 2;

q is 0, 1,2, 3, or 4;

each R is independently selected from the group consisting of halogen, -OH, $-NH_2$, $-NO_2$, -CN, $-CF_3$, -OCF3, $-N_3$, $-SO_3H$, $-S(=O)_2$alkyl, -S(-O)alkyl, $-OS(=O)_2CF_3$, acyl, -O-acyl, alkyl, alkoxyl, alkylamino, alkylarylamino, alkylthio, cycloalkyl, alkylaryl, aryl, heteroaryl, heterocyclyl, heterocyclylalkyl, alkenyl, alkynyl, (hetero-)aryl, (hetero-)arylthio, and (hetero-)arylamino; wherein each acyl, -O-acyl, alkyl, alkoxyl, alkylamino, alkylarylamino, alkylthio, cycloalkyl, alkylaryl,, aryl, heteroaryl, heterocyclyl, heterocyclylalkyl, alkenyl, alkynyl, (hetero-)aryl, (hetero-)arylthio, and (hetero-)arylamino may be substituted or unsubstituted;

R_1 is selected from the group consisting of H, oxo, alkyl, alkenyl, aryl, alkylaryl, cycloalkyl, heteroaryl, and heterocyclyl; wherein each alkyl, alkenyl, aryl, alkylaryl, cycloalkyl, heteroaryl, and heterocyclyl may be substituted or unsubstituted;

R_2 is selected from the group consisting of H, $-C(=O)R_5$, $-C(=S)R_6$, $-SO_2R_7$, $-P(=O)R_8R9$, $-(CH_2)_m-R_{10}$, alkyl, aryl, alkylaryl, heteroaryl, cycloalkyl, cycloalkylalkyl, and heterocyclyl; wherein each alkyl, aryl, alkylaryl, heteroaryl, cycloalkyl, cycloalkylalkyl, and heterocyclyl may be substituted or unsubstituted;

R_3 is selected from the group consisting of H, $-CO_2Y$, -C(=O)NHY, acyl, -O-acyl, alkyl, alkenyl, aryl, alkylaryl, cycloalkyl, heteroaryl, and heterocyclyl; wherein each acyl, alkyl, alkenyl, aryl, alkylaryl, cycloalkyl, heteroaryl, and heterocyclyl may be substituted or

unsubstituted; and wherein Y is selected from the group consisting of H, alkyl, aryl, alkylaryl, cycloalkyl, heteroaryl, and heterocyclyl, and wherein each alkyl, aryl, alkylaryl, cycloalkyl, heteroaryl, and heterocyclyl may be substituted or unsubstituted;

R_4 is selected from the group consisting of H, alkyl, alkenyl, aryl, alkylaryl, cycloalkyl, heteroaryl, and heterocyclyl; wherein each alkyl, alkenyl, aryl, alkylaryl, cycloalkyl, heteroaryl, and heterocyclyl may be substituted or unsubstituted;

R_5 is selected from the group consisting of-$NR_{15}R_{16}$, -$(CH_2)zNR_{15}R_{16}$, -$NHNR_{15}R_{16}$> -NHOH, - OR_{15}, -$C(O)NHNR_{15}R_{16}$, -CO_2R_{15}, -$C(=O)NR_{15}R_{16}$, -CH_2X, acyl, alkyl, alkenyl, aryl, alkylaryl, cycloalkyl, cycloalkylalkyl, heteroaryl, heterocyclyl, and heterocyclylalkyl; wherein each acyl, alkyl, alkenyl, aryl, alkylaryl, cycloalkyl, cycloalkylalkyl, heteroaryl, heterocyclyl, and heterocyclylalkyl may be substituted or unsubstituted, and wherein z is 1, 2, 3, 4, 5, or 6;

R_6 is selected from the group consisting of-OR15, -$NHNR_{]5}R_{16}$, -NHOH, -NRi_5Ri_6, -CH_2X, acyl, alkenyl, alkyl, aryl, alkylaryl, cycloalkyl, cycloalkylalkyl, heteroaryl, heterocyclyl, and heterocyclylalkyl; wherein each acyl, alkenyl, alkyl, aryl, alkylaryl, cycloalkyl, cycloalkylalkyl, heteroaryl, heterocyclyl, and heterocyclylalkyl may be substituted or unsubstituted;

R_7 is selected from the group consisting of-OR_{15}, -$NR_{15}R_{16}$, -$NHNR_{15}R_{16}$, -NHOH, -CH_2X, alkyl, alkenyl, alkynyl, aryl, alkylaryl, cycloalkyl, cycloalkylalkyl, heteroaryl, heterocyclyl, and heterocyclylalkyl; wherein each alkyl, alkenyl, alkynyl, aryl, alkylaryl, cycloalkyl, cycloalkylalkyl, heteroaryl, heterocyclyl, and heterocyclylalkyl may be substituted or unsubstituted;

R_8 and R_9 independently are selected from the group consisting of OH, acyl, alkenyl, alkoxyl, alkyl, alkylamino, aryl, alkylaryl, cycloalkyl, cycloalkylalkyl, heteroaryl, heterocyclyl, and heterocyclylalkyl; wherein each acyl, alkenyl, alkoxyl, alkyl, alkylamino, aryl, alkylaryl, cycloalkyl, cycloalkylalkyl, heteroaryl, heterocyclyl, and heterocyclylalkyl may be substituted or unsubstituted;

R_{10} is selected from the group consisting of -$NR_{15}R_{16}$, OH, -SO_2R_{11}, -$NHSO_2R_{11}$, $C(=O)(R_{12})$, $NHC=O(R_{12})$, -$OC=O(R_{12})$, and -$P(=O)R_{13}R_{14}$;

R_{11}, R_{12}, R_{13}, and R_{14} independently are selected from the group consisting of H, OH, NH_2, - $NHNH_2$, -NHOH, acyl, alkenyl, alkoxyl, alkyl, alkylamino, aryl, alkylaryl, cycloalkyl, cycloalkylalkyl, heteroaryl, heterocyclyl, and heterocyclylalkyl; wherein each acyl, alkenyl,

alkoxyl, alkyl, alkylamino, aryl, alkylaryl, cycloalkyl, cycloalkylalkyl, heteroaryl, heterocyclyl, and heterocyclylalkyl may be substituted or unsubstituted;

X is selected from the group consisting of halogen, -CN, $-CO_2R_{15}$, $-C(=O)NR_{15}R_{16}$, $-NR_{15}R_{16}$, $-OR_{15}$, $-SO_2R_7$ and $-P(=O)R_8R_9$;

R_{15} and R_{16} independently are selected from the group consisting of H, acyl, alkenyl, alkoxyl, OH, NH_2, alkyl, alkylamino, aryl, alkylaryl, cycloalkyl, cycloalkylalkyl, heteroaryl, heterocyclyl, and heterocyclylalkyl; wherein each acyl, alkenyl, alkoxyl, alkyl, alkylamino, aryl, alkylaryl, cycloalkyl, cycloalkylalkyl, heteroaryl, heterocyclyl, and heterocyclylalkyl may be substituted or unsubstituted; and optionally R_{15} and R_{16} together with the N to which they are bonded may form a heterocycle which may be substituted; and

the nitrogen in the benzothiazepine ring may optionally be a quaternary nitrogen, and enantiomers, diastereomers, tautomers, pharmaceutically acceptable salts, hydrates, solvates, complexes, and prodrugs thereof.

[0066] It should be appreciated that the above 1,4-benzothiazepine derivative compounds are provided as an example only and do not limit the compounds identified by the present methods. It would also be appreciated that the present identifying and screening methods are not limited by the structure of the compounds and can be used to screen diverse libraries of compounds as desired.

[0067] *In vivo* screening methods

[0068] In certain aspects, the invention provides *in vivo* methods for determining the composition and characteristics of the RyR complex, for example but not limited to the phosphorylation state, in an animal. In certain embodiments, the animal is suffering from a disease or disorders associated with RyR receptor complex, such as a cardiac, skeletal muscle, or neurological disease or disorder associated with abnormal structure or function of the RyR complex. In certain embodiments, the methods comprise measuring a change in the binding of a calstabin to a ryanodine receptor. In one embodiment, the method comprises administering to an animal a physiologically effective amount of a compound that regulates calstabin binding to a ryanodine receptor and measuring the calstabin binding to the ryanodine receptor in a blood sample from the animal. The animal can be a mammal, including a human, but also other animals, especially mammals such as mice, rats, dogs, and pigs can be used. In an example, the measurement of calstabin binding to RyR in an animal

treated with the compound, i.e., an animal to which the compound is administered, is compared with calstabin binding to RyR in an untreated animal, i.e., an animal to which the compound is not administered.

[0069] In another embodiment, the invention provides *in vivo* methods for identifying compounds that modulate binding of a calstabin to a ryanodine receptor and that can be used to treat diseases associated with RyRs. Generally, the methods involve administering to an animal, e.g., a mouse or another mammal such as a rat, a dog, or a pig, a physiologically effective amount of a regulating compound that regulates or decreases calstabin binding to a ryanodine receptor and then measuring calstabin binding to such receptor in that animal; administering to another, similar animal the same amount of the regulating compound in combination with an amount of a test compound and measuring calstabin binding to a ryanodine receptor in that animal; and comparing the measured calstabin binding in a blood sample from each animal to determine whether the test compound changes or increases calstabin binding to the ryanodine receptor. The difference in binding of the calstabin to the ryanodine receptor between the animals indicates whether and to what extent the test compound modulates binding of the calstabin to the ryanodine receptor. The administration and measurements in each animal can be made in any desired order, sequentially or at the same time, and by any mode of administration (e.g., oral or subcutaneous via an osmotic pump).

[0070] Advantageously, the present methods can be used to screen, *in vivo*, a plurality of compounds to identify a compound that modulates binding of a calstabin to a ryanodine receptor, by administering the regulating compound and the test compound to a plurality of animals and comparing the calstabin binding measurement in each of these animals to that measured from an animal that received only the regulating compound. For example, multiple forms of a compound can be tested in separate animals or groups of animals, together with the appropriate positive and negative controls, such that overall comparison of calstabin levels in various forms of a compound can be made in a simple and effective manner.

[0071] Regulatory/Regulating Compounds

[0072] The compound that regulates calstabin binding to a ryanodine receptor can be any agent that can regulates RyR function *in vivo*. In certain embodiments, the regulating compound is capable of decreasing calstabin binding to RyR. In an example, the regulating

compound is able to disrupt calstabin-2 binding to RyR2, especially in cardiac muscle tissue. In another example, the regulating compound is able to disrupt calstabin-1 binding to RyRl, especially in skeletal muscle tissue. In yet another example, the regulating compound is able to disrupt calstabin-1 binding to RyR3, especially in the brain.

[0073] In a further embodiment, the compound is a beta-agonist, such as the synthetic beta-agonist, e.g., isoproterenol. Upon stimulation, beta-adrenergic receptors trigger signaling pathways that can lead to decreased calstabin binding to RyR. Without wishing to be bound by any particular mechanism or theory, in cardiac muscle cells, for example, this pathway involves G protein-coupled receptors that activate adenylyl cyclase, inducing production of the second messenger cyclic AMP, which in turn can activate protein kinase A (PKA). PKA then hyperphosphorylates RyR channels, decreasing its affinity for the channel stabilizer calstabin. Thus, according to the invention, regulating compounds include agents that modulate steps in the beta-adrenergic signaling pathway. In an example, the compound is an activator of G-proteins or a stimulator of PKA, such as forskolin.

[0074] The regulating compound is administered to a subject by contacting target tissue cells *(e.g.,* cardiac cells) *in vivo.* In an example, the regulatory compound contacts the tissue directly, for example, by binding to a surface receptor or by stimulating adenylate cyclase. Alternatively, the regulating compound can contact the tissue indirectly. For instance, the regulating compound itself triggers beta-adrenergic signaling by mimicking the action of preganglionic cells in the SNS, and thereby activates beta-adrenergic signaling through the sympathetic postganglionic fibers that innervate target organs.

[0075] In one embodiment, the regulating compound is administered to the subject in a physiologically effective amount that can disrupt the interaction between calstabin and RyR, e.g., the interaction between calstabin-2 and RyR2. The effective amount can be readily determined by the skilled artisan based on standard criteria, such as the delivery method, administration route, established titration studies, and methods and assays disclosed herein. When the compound is delivered to the subject via blood, its concentration in the blood over time can be determined by removing samples and analyzing the levels in the plasma. In an example, the regulating compound is a beta agonist, for e.g. isoproterenol, and has an effective amount of approximately 0.5 mg/kg/hr, when administered by an osmotic pump that has been implanted subcutaneously in a mouse. In certain embodiments, the beta agonist is chronically administered to the subject, for example, for three to five days.

[0076] As contemplated herein, the applicants have found that administering the regulatory compound subcutaneously in an osmotic pump for at least about 3 days (e.g., for about 5 days) results in a biological effect such as phosphorylation of RyR and concomitant decreased binding of calstabin to RyR.

[0077] Calstabin Binding to RyR

[0078] In one embodiment, *in vivo* binding of calstabin to RyR can be determined by obtaining a blood sample which contains lymphocytes, making a buffy coat preparation therefrom, immunoprecipitating RyR complexes from the sample, and analyzing the complexes for the presence of calstabin. Levels of phosphorylated-RyR and total RyR can also be determined.

[0079] Protein levels, particularly the level of RyR-bound calstabin, can be assayed in a blood or buffy coat sample by standard methods and techniques, including those readily determined from the art (e.g., immunological techniques, hybridization analysis, Immunoprecipitation, Western-blot analysis, fluorescence imaging techniques, and/or radiation detection, etc.), as well as any assays and detection methods disclosed herein.

[0080] Test Compounds

[0081] Test compounds include any agent that hasan effect on, or is to be tested to determine if it has an effect on, RyR channel function, and more particularly, on the interaction of RyR with calstabin. Test compounds include chemicals, small molecules, proteins, peptides, antibodies, and RNA-regulating agents, including those based on antisense, ribozyme, and RNAi technologies.

In an embodiment, the test compounds are 1,4-benzothiazepine derivatives represented by Formula I, as described above.

[0082] For the *in vivo* screening methods described herein, test compounds may be administered to the subject in an amount effective to change, limit or prevent a decrease in, or increase, the level of RyR-bound calstabin in the subject, particularly, in cells of the subject. The effective amount is readily determined by a skilled artisan, based on known procedures, consideration of the administrative routes, analysis of titration curves, and methods and assays disclosed herein. For example, a suitable amount of compounds having or based on a 1,4-benzothiazepine structure ranges from about 5 mg/kg/day to about 20

mg/kg/day, and/or is an amount sufficient to achieve plasma levels ranging from about 5 ng/ml to about 1000 ng/ml. In a further example, the amount of a compound of Formula I ranges from about 10 mg/kg/day to about 20 mg/kg/day.

[0083] In certain embodiments, the test compound is administered as is, without further processing. If desired, however, the compound can be administered in a pharmaceutical formulation, e.g., in admixture with a suitable pharmaceutical excipient, diluent, or carrier selected with regard to the intended route of administration and standard pharmaceutical practices. Accordingly, in one aspect, the invention provides a pharmaceutical composition or formulation comprising at least one test compound, or a pharmaceutically acceptable derivative thereof, as the active ingredient in association with a pharmaceutically acceptable excipient, diluent and/or carrier that is compatible with the other ingredients of the formulation and that is not deleterious to the recipient thereof.

[0084] According to certain embodiments, the test compound modulates binding of a calstabin to a ryanodine receptor by increasing the binding of the calstabin to the RyR. In an example, the test compound is able to increase binding of calstabin-2 to RyR2, especially in cardiac muscle tissue. In another example, the test compound is able to increase binding of calstabin-1 to RyRl, especially in skeletal muscle tissue. In still another example, the test compound is able to increase binding of calstabin-1 binding to RyR3, especially in the brain. Therefore, the test compound can limit or prevent decreased levels of RyR-bound calstabin in cells of a subject, or increase such levels, by increasing the ability of calstabin to bind to phosphorylated RyR.

[0085] Administering of Compounds

[0086] Regulating compounds and test compounds are administered to a subject by contacting target tissues cells, e.g., cardiac muscle cells, *in vivo*, using any suitable technique, including known techniques used to introduce and administer proteins, nucleic acids, small molecules, and other drugs. Examples of methods of administering the compound include, without limitation, absorption, electroporation, immersion, injection, liposome delivery, transfection, transfusion, vectors and other drug-delivery vehicles and other methods, including oral delivery and subcutaneous administration. When administration to a particular tissue or portion of the subject is desired, e.g., when target cells are localized to a particular tissue or portion of a subject, it is desirable to introduce the

compounds directly to the target tissue or portion, by injection or by some other suitable means (e.g., by introducing the compounds into the blood or another body fluid). Such means include intravenous, intra-arteriole, intramuscular, intradermal, subcutaneous, intraperitoneal, transmucosal, and transdermal routes.

[0087] In certain embodiments, the compound is administered to the subject subcutaneously. For example, the compound is administered to the subject via an implanted osmotic infusion pump that contains the compound. The osmotic pump is implanted subcutaneously, allowing the compound to target multiple tissues in the body through the bloodstream. If desired, plasma samples can be isolated over time and analyzed to determine the concentration of the compound in the blood. Other configurations for osmotic pump infusion are also contemplated. For example, the pump can be implanted intraperitoneally or connected to a catheter to target the compounds to a specific tissue or organ by microperfusion. Such targets include the venous or arterial systems, brain, muscles, spinal cord, or other organs and tissues. Osmotic pumps can also be implanted serially if a longer period of perfusion or microperfusion is desired. Thus, osmotic pumps containing a regulating or test compound provide a versatile means for inducing a measurable change in calstabin binding to RyR in any suitable animal system. Osmotic pump implantation can be used with various animals, including fish, frogs, birds, and many mammals, including rats, guinea pigs, rabbits, cats, dogs, pigs, goats, sheep, horses, and primates, including humans.

[0088] In another embodiment, the compound, especially the test compound, is administered orally. For example, when orally administering to small animals, the compound can be administered by gavage feeding. Oral administration is especially convenient and preferred when administering the compound to humans.

[0089] In yet another aspect, the regulating compound and the test compound can be administered by the same or different routes, as desired. For example, the regulating compound can be chronically administered by an implanted osmotic pump, and the test compound can be delivered by oral administration. Alternatively, both the regulating compound and the test compound can be administered by an implanted osmotic pump.

[0090] When orally administered, the compound (e.g., the test compound) can be administered by any dosing regimen known in the art, including, but not limited to once daily, twice daily or thrice daily. In addition, the test compound can be administered

concurrently with the regulatory compound (e.g., for 3, 4 or 5 days, by any mode of administration), or it can be administered before of after the regulatory compound. In one embodiment, the test compound is administered following administration of the regulatory compound for a time period sufficient to elicit a biological effect such as RyR phosphorylation and concomitant decrease in calstabin binding to RyR.

Monitoring Disease Progression

[0091] The present invention also provides a method of monitoring the progression of a disease or disorder associated with abnormal ryanodine receptor structure or function comprising: (a) obtaining a first blood sample from a test subject, (b) obtaining a second blood sample from the test subject, wherein the second sample is obtained after the first sample, (c) determining the amount of a calstabin protein bound to a ryanodine receptor protein in the first sample and the second sample; and (d) comparing the amount of the calstabin protein bound to the ryanodine receptor in the first and second sample, whereby a lower amount of calstabin protein bound to the ryanodine receptor in the second sample as compared to the first sample indicates progression of the disease or diosrder in the subject. Variations on this method will be apparent to those skilled in the art and can be used to monitoring the progression of a disease or disorder associated with abnormal ryanodine receptor structure or function.

Monitoring Drug Efficacy

[0092] The present invention also provides a method of monitoring drug efficacy in a subject comprising: (a) obtaining a first blood sample from a test subject who has a disease or disorder associated with abnormal ryanodine receptor, (b) administering a drug to said subject, (c) obtaining a second blood sample from the test subject, (d) determining the amount of a calstabin protein bound to a ryanodine receptor protein in the first sample and the second sample; and (e) comparing the amount of the calstabin protein bound to the ryanodine receptor in the first and second sample, whereby a higher amount of calstabin protein bound to the ryanodine receptor in the second sample as compared to the first sample is indicative of the efficacy of the drug in increasing theamount of calstabin protein bound to the ryanodine receptor. The efficacy of the drug in increasing the amount of calstabin protein bound to the ryanodine receptor may be indicative of efficacy in treating the disease or disorder associated with abnormal ryanodine receptor complex structure or function. It should be noted that the term "efficacy" as used herein, refers to any detectable increase in binding of calstabin to the

ryanodine receptor and/or any detectable improvement or lessening of any symptom of disease or disorder associated with abnormal ryanodine receptor complex structure or function.

Incorporation by Reference

[0093] Throughout this application, various other publications, patents, and patent applications are referenced. The disclosures of these documents in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art as known to those skilled therein as of the date of the invention described and claimed herein.

EXAMPLES

[0094] The following examples are exemplary only, and do not limit the scope of the invention or the appended claims.

EXAMPLE 1

Effect of 1,4, benzothiazepine RyCal compounds on Calstabin Binding in Lymphocytes

[0095] Animals and Reagents

[0096] Rats were maintained and studied according to approved protocols. The synthetic beta-adrenergic agonist, isoproterenol (ISO) was obtained from Sigma (I65627) and prepared as a 100 mg/ml stock in water. Lysis buffer was made by adding sucrose (1 mM), dithiothreitol (320 mM), and 1 protease inhibitor tablet (10X) to 10 ml stock solution (10 mM HEPES, 1 mM EDTA, 20 mM NaF, 2 mM Na3VO4).

[0097] Osmotic Pump Preparation and Surgical Implantation

[0098] Rats were continuously infused for five days with 10 mg/ml isoproterenol (10 μl/hr) by means of a subcutaneously implanted osmotic infusion pump (Alzet MiniOsmotic pump, Model 2001, Durect Corporation, Cupertino, CA).

[0099] For isoproterenol (ISO) drug loading, the osmotic pump was held vertically and 200 μl drug solution was injected into the pump via a 2 ml syringe (attached to a cannula) that

contained an excess of drug solution (2.2ml per pump). The drug solution was injected slowly downward, while the syringe was slowly lifted, until the pump was overfilled. Overflow of displaced fluid upon capping the pump confirmed that the pump was properly filled.

[00100] The loaded osmotic pumps were implanted subcutaneously by the following steps. The recipient rat was anesthetized with 1.5-2% isofluorane in O2 administered at 0.6 L/min, and its weight was then measured and recorded. The rat was then placed chest-down on styrofoam, its face in the nose cone. The fur was clipped on the back of the neck, extending behind the ears to the top of the head. The area was wiped gently with 70% alcohol, and a small incision was made at the midline on the nape of head/neck. A suture holder was swabbed with alcohol, inserted into the cut, and opened to release the skin from the underlying tissue. To accommodate the pump, this opening was extended back to the hindquarters. The loaded pump was inserted into the opening, with its release site positioned away from the incision, and was allowed to settle underneath the skin with minimal tension. The incision was closed with 5.0 nylon suture, requiring about 5-6 sutures, and the area was wiped gently with 70% alcohol. Following surgery, mice were placed in individual cages to minimize injury and possible activation of the sympathetic nervous system.

[00101] Drug Treatment

[00102] Rats divided into 4 groups, Control, isoproterenol ("ISO") treated (via osmotic pump as described above), ISO treated + ARM036 (a single dose of 2 mg by oral gavage on day 5), and ISO treated + ARM064 ((a single dose of 2 mg by oral gavage on day 5). ARM036 and ARM064 are 1,4, benzothiazepines, the structures of which are provided in published PCT application WO 07/024717 (PCT/US06/32405). In the following description, these compounds are referenced with a corresponding compound number, in which the prefixes "S" and "ARM" are used interchangeably. For example, compound "S36" is the same compound "ARM036" and compound "S64" is the same as "ARM064."

[00103] Preparation of Buffy Coat Samples

[00104] Whole blood (10 ml) was collected from individual rats from the four groups desctribed above. Blood samples were centrifuged at 2,000 x g for 10 minutes. After removing plasma, the buffy coat was carefully isolated and layered on 2.0 mls of lymphocyte

separation buffer (Fisher Scientific). Samples were centrifuged for 2,500 x g for 20 minutes. Lymphocytes are isolated and washed 2 x with PBS (2,500 x g for 10 minutes). Lymphocytes from 4 rats were combined and lysed in 0.25 ml RIPA Buffer (50 mM Tris-HCl pH 7.4, 0.9% NaCl, 5.0 mM NaF, 1.0 mM Na3VO4, 1.0% Triton-X100, and protease inhibitors) by passing through a 20 gauge needle 20 times. Samples were centrifuged at 2,500 x g to remove cellular debris. The supernatant was divided into 4 equal parts. The sample was resuspend in a total of 0.5 ml of RIPA buffer. RyR1, RyR2, or RyR3 proetins are then immunoprecipitated using isoform specific antibodies (1:250 dilution), and the immunoprecipitates are analyzed for total RyR, PKA phosphorylated RyR, and calstabin using IR labeled secondary antibodies and the Odyssey Infrared Imaging System (LICOR Biosciences, Lincoln NE).

[00105] RyR Immunoprecipitation from Buffy Coat Samples

[00106] The ryanodine receptor was immunoprecipitated from samples by incubating 200-500 μg of homogenate with 2 μl anti-RyR antibody (RyR2-5029; Jayaraman et al., J. Biol. Chem. 1992;267:9474-77) in 0.5 ml of a modified RIPA buffer (50 mM Tris-HCl (pH 7.4), 0.9% NaCl, 5.0 mM NaF, 1.0 mM Na3VO4, 0.5% Triton-X100, and protease inhibitors) at 4°C for 1.5 hr. The samples were then incubated with Protein A sepharose beads (Amersham Pharmacia Biotech, Piscatawy, NJ) at 4°C for 1 hour, after which the beads were washed three times with RIPA. Samples were heated to 95°C and size fractionated by SDS-PAGE (6% for SDS-PAGE RyR and 15% SDS-PAGE for calstabin). Immunoblots were developed using an anti-RyR antibody (RyR2-5029) at a 1:5,000 dilution, a phospho-specific antibody (RyR2-P2809, Zymed Laboratories, San Francisco, CA) at a 1:10,000 dilution or an anti-FKBP antibody (FKBB12/12/6, Jayaraman et al., J. Biol. Chem. 1992;267:9474-77) at a 1:2,000 dilution. The antibodies were diluted in 5% milk or TBS-T (20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 0.05% Tween® 20, 0.5% Triton X-100).

[00107] Results

[00108] As shown in FIG. 1, isoproterenol treatment alone increased RyR phosphorylation in the rat lymphocytes, as can be seen by comparing the "Control" (no ISO) lane with the "Vehicle" (ISO but no test compound) lane in each panel. Further, isoproterenol treatment alone led to decreased calstabin levels. Notably, test compounds

S36 and S64 led to enhanced calstabin binding to both RyR1 and RyR2 in the lymphocyte/buffy coat samples.

CLAIMS

What is claimed is:

1. A method of diagnosing a disease or disorder associated with abnormal ryanodine receptor function in a test subject comprising:
   (a) obtaining a blood sample from a test subject,
   (b) determining the amount of a calstabin protein bound to a ryanodine receptor protein in the blood sample from the test subject, and
   (c) comparing the amount of the calstabin protein bound to the ryanodine receptor in the test subject to a control measurement for the amount of the calstabin protein bound to the ryanodine receptor,
   whereby a lower amount of calstabin protein bound to the ryanodine receptor in the test subject as compared to the control measurement indicates that the test subject may have a disease or disorder associated with abnormal ryanodine receptor function.

2. A method of diagnosing a disease or disorder associated with abnormal ryanodine receptor comprising:
   (a) obtaining a blood sample from a test subject,
   (b) determining the amount of a calstabin protein bound to a ryanodine receptor protein in the sample from the test subject, and
   (c) comparing the amount of the calstabin protein bound to the ryanodine receptor in the test subject to to the amount of calstabin protein bound to the ryanodine receptor in a control sample obtained from a control subject known to have normal ryanodine receptor protein;
   whereby a lower amount of calstabin protein bound to the ryanodine receptor in the test subject as compared control indicates that the test subject has or is at risk of developing a disease or disorder associated with abnormal ryanodine receptor function.

3. The method of claim 1, wherein the control measurement is determined by:
   (a) obtaining a tissue sample containing a ryanodine receptor and a calstabin protein from a control subject known to have normal ryanodine receptor function, and
   (b) determining the amount of the calstabin protein bound to the ryanodine receptor protein in the tissue sample from the control subject,

thereby giving a control measurement for the amount of the calstabin protein bound to the ryanodine receptor in a subject known to have normal ryanodine receptor function.

4. The method of claim 1, wherein the control measurement is obtained from a prior measurement of the the amount of calstabin protein bound to ryanodine receptor obtained using a ryanodine receptor known to have normal function and normal interaction with calstabin.

5. The method of claim 1 or 2, wherein the blood sample is a peripheral blood sample.

6. The method of claim 1 or 2, wherein the blood sample contains lymphocytes.

7. The method of claim 6, wherein the lymphocytes comprise B cells.

8. The method of claim 6, wherein the lymphocytes comprise T cells.

9. The method of claim 1 or 2, wherein the calstabin protein is selected from the group consisting of calstabin 1 and calstabin 2.

10. The method of claim 1 or 2, wherein the ryanodine receptor protein is selected from the group consisting of RyR1, RyR2, and RyR3.

11. The method of claim 1 or 2, wherein determining the amount of the calstabin protein bound to the ryanodine receptor protein is performed by immunoprecipating the ryanodine receptor protein and detecting, and quantifying the amount of, the calstabin protein present in the immunoprecipated ryanodine receptor protein complex.

12. The method of claim 1 or 2, wherein determining the amount of the calstabin protein bound to the ryanodine receptor protein is performed by immunoprecipating the calstabin protein complex and detecting, and quantifying the amount of, the ryanodine receptor protein present in the immunoprecipated calstabin protein complex.

13. The method of claim 11, wherein the ryanodine receptor protein complex is immunoprecipated using an anti-ryanodine receptor antibody and the calstabin protein is detected using an anti-calstabin antibody.

14. The method of claim 12, wherein the calstabin protein complex is immunoprecipated using an anti-calstabin antibody and the ryanodine receptor protein is detected using an anti-ryanodine receptor antibody.

15. The method of claim 1 or 2, wherein the ryanodine receptor or calstabin is labeled, and the amount of ryanodine receptor bound to calstabin is determined by detecting and quantifying the label.

16. The method of claim 15, wherein label is a fluorescent label or a radiolabel.

17. The method of claim 1 or 2, wherein the test subject is a mammal.

18. The method of claim 1 or 2, wherein the test subject is a human.

19. The method of claim 2 or 3, wherein the control subject is a mammal.

20. The method of claim 2 or 3, wherein the control subject is a human.

21. The method of claim 1 or 2, wherein the disease or disorder is selected from the group consisting of cardiac disorders and diseases, skeletal muscular disorders and diseases, cognitive disorders and diseases, malignant hyperthermia, diabetes, and sudden infant death syndrome.

22. The method of claim 21, wherein the cardiac disorders and diseases are selected from the group consisting of irregular heartbeat disorders and diseases, exercise-induced irregular heartbeat disorders and diseases, sudden cardiac death, exercise-induced sudden cardiac death, congestive heart failure, chronic obstructive pulmonary disease, and high blood pressure.

23. The method of claim 22, wherein the irregular heartbeat disorders and diseases are selected from the group consisting of atrial and ventricular arrhythmias, atrial and ventricular fibrillation, atrial and ventricular tachyarrhythmias, atrial and ventricular tachycardias, catecholaminergic polymorphic ventricular tachycardia (CPVT), and exercise-induced variants thereof.

24. The method of claim 21, wherein the skeletal muscular disorders and diseases are selected from the group consisting of skeletal muscle fatigue, exercise-induced skeletal muscle fatigue, myopathies, muscular dystrophy, bladder disorders, and incontinence.

25. The method of claim 21, wherein the cognitive disorders and diseases include are selected from the group consisting of Alzheimer's Disease, memory disorders, and age-dependent memory disorders.

26. A method for detecting a mutation in a ryanodine receptor in a test subject comprising:
(a) obtaining a sample of blood from the subject,
(b) obtaining a DNA sample from the blood sample,
(c) determining the nucleotide sequence of the DNA encoding a ryanodine receptor in the DNA sample from the test subject, and
(d) comparing the nucleotide sequence of the ryanodine receptor in the test subject to the nucleotide sequence of a corresponding wild type ryanodine receptor, whereby a difference between the nucleotide sequence of the DNA encoding the ryanodine receptor in the test subject and the nucleotide sequence of the corresponding wild type ryanodine receptor, indicates that the test subject has a mutation in the gene encoding the ryanodine receptor.

27. The method of claim 26, wherein the blood sample is a peripheral blood sample.

28. The method of claim 26, wherein the blood sample contains lymphocytes.

29. The method of claim 28, wherein the lymphocytes comprise B cells.

30. The method of claim 28, wherein the lymphocytes comprise T cells.

31. The method of claim 26, wherein the DNA sample comprises genomic DNA.

32. The method of claim 26, wherein the DNA sample comprises cDNA.

33. The method of claim 26, wherein the nucleotide sequence of the wild type ryanodine receptor is obtained from a public nucleotide sequence database.

34. The method of claim 26, wherein the ryanodine receptor protein is selected from the group consisting of RyR1, RyR2, and RyR3.

35. The method of claim 26, wherein the test subject is a mammal.

36. The method of claim 26, wherein the test subject is a human.

37. The method of claim 26, wherein the mutation is associated with a disease or disorder is selected from the group consisting of cardiac disorders and diseases, skeletal muscular disorders and diseases, cognitive disorders and diseases, malignant hyperthermia, diabetes, and sudden infant death syndrome.

38. The method of claim 37, wherein the cardiac disorders and diseases are selected from the group consisting of irregular heartbeat disorders and diseases, exercise-induced irregular heartbeat disorders and diseases, sudden cardiac death, exercise-induced sudden cardiac death, congestive heart failure, chronic obstructive pulmonary disease, and high blood pressure.

39. The method of claim 38, wherein the irregular heartbeat disorders and diseases are selected from the group consisting of atrial and ventricular arrhythmias, atrial and ventricular fibrillation, atrial and ventricular tachyarrhythmias, atrial and ventricular tachycardias, catecholaminergic polymorphic ventricular tachycardia (CPVT), and exercise-induced variants thereof.

40. The method of claim 37, wherein the skeletal muscular disorders and diseases are selected from the group consisting of skeletal muscle fatigue, exercise-induced skeletal muscle fatigue, myopathies, muscular dystrophy, bladder disorders, and incontinence.

41. The method of claim 37, wherein the cognitive disorders and diseases include are selected from the group consisting of Alzheimer's Disease, memory disorders, and age-dependent memory disorders.

42. A method of determining the amount of ryanodine receptor phosphorylation in a test subject comprising:
(a) obtaining a sample of blood from a test subject,
(b) determining the amount of a phosphorylation of a ryanodine receptor protein in the blood sample,
(c) comparing the amount of phosphorylation of the ryanodine receptor in the test subject to a control measurement of the amount of phosphorylation of a ryanodine receptor,
whereby a higher amount of the ryanodine receptor phosphorylation in the test subject as compared to the control measurement indicates that the test subject has or is at risk of developing a ryanodine receptor hyperphosphorylation defect and has or is at risk of developing, a disease or disorder associated with ryanodine receptor hyperphosphorylation.

43. A method of detecting the level of ryanodine receptor phosphorylation in a test subject comprising:
(a) obtaining a sample of blood from a test subject,
(b) determining the amount of a phosphorylation of a ryanodine receptor protein in the a sample of blood from the test subject;
and
(c) comparing the amount of phosphorylation of the ryanodine receptor in the test subject to the amount of phosphorylation of a ryanodine receptor in a control sample obtained from a control subject known to have normal ryanodine phosphorylation,
whereby a higher amount of the ryanodine receptor phosphorylation in the test subject as compared control indicates that the test subject has or is at risk of

developing a ryanodine receptor hyperphosphorylation defect and has or is at risk of developing, a disease or disorder associated with ryanodine receptor hyperphosphorylation.

44. The method of claim 42, wherein the control measurement is determined by:
(a) obtaining a tissue sample containing a ryanodine receptor from a control subject known to have normal ryanodine receptor phosphorylation, and
(b) determining the amount of phosphorylation of the ryanodine receptor protein in the tissue sample from the control subject,
thereby giving a control measurement for the amount of phosphorylation of the ryanodine receptor in a subject known to have normal ryanodine receptor phosphorylation.

45. The method of claim 42, wherein the control measurement is obtained from a prior measurement of the the amount of phosphorylation of the ryanodine receptor obtained using a ryanodine receptor known to have normal phosphorylation.

46. The method of claim 42 or 43, wherein the blood sample is a peripheral blood sample

47. The method of claim 42 or 43, wherein the blood sample contains lymphocytes.

48. The method of claim 47, wherein the lymphocytes comprise B cells.

49. The method of claim 47, wherein the lymphocytes comprise T cells.

50. The method of claim 42 or 43, wherein the ryanodine receptor protein is selected from the group consisting of RyR1, RyR2, and RyR3.

51. The method of claim 42 or 43, wherein the test subject is a mammal.

52. The method of claim 42 or 43, wherein the test subject is a human.

53. The method of claim 43 or 44, wherein the control subject is a mammal.

54. The method of claim 43 or 44, wherein the control subject is a human.

55. The method of claim 42 or 43, wherein the disease or disorder is selected from the group consisting of cardiac disorders and diseases, skeletal muscular disorders and diseases, cognitive disorders and diseases, malignant hyperthermia, diabetes, and sudden infant death syndrome.

56. The method of claim 55, wherein the cardiac disorders and diseases are selected from the group consisting of irregular heartbeat disorders and diseases, exercise-induced irregular heartbeat disorders and diseases, sudden cardiac death, exercise-induced sudden cardiac death, congestive heart failure, chronic obstructive pulmonary disease, and high blood pressure.

57. The method of claim 56, wherein the irregular heartbeat disorders and diseases are selected from the group consisting of atrial and ventricular arrhythmias, atrial and ventricular fibrillation, atrial and ventricular tachyarrhythmias, atrial and ventricular tachycardias, catecholaminergic polymorphic ventricular tachycardia (CPVT), and exercise-induced variants thereof.

58. The method of claim 55, wherein the skeletal muscular disorders and diseases are selected from the group consisting of skeletal muscle fatigue, exercise-induced skeletal muscle fatigue, myopathies, muscular dystrophy, bladder disorders, and incontinence.

59. The method of claim 55, wherein the cognitive disorders and diseases include are selected from the group consisting of Alzheimer's Disease, memory disorders, and age-dependent memory disorders.

60. A method for identifying a chemical compound that enhances binding of a calstabin protein to a ryanodine receptor protein, or inhibits dissociation of a calstabin protein from a ryanodine receptor protein, comprising
(a) obtaining a first blood sample,
(b) contacting the first blood sample with an agent that causes dissociation of a calstabin protein from a ryanodine receptor protein,

(c) measuring the amount of a calstabin protein bound to a ryanodine receptor protein in the first blood sample,
(d) obtaining a second blood sample,
(e) contacting the second blood sample with an agent that causes dissociation of a calstabin protein from a ryanodine receptor protein and a test compound,
(f) measuring the amount of the calstabin protein bound to the ryanodine receptor protein in the second blood sample, and
(g) comparing the amount of the calstabin protein bound to the ryanodine receptor protein in the first and second blood samples,
whereby a higher amount of calstabin protein bound to the ryanodine receptor protein in the second sample as compared to the first sample indicates that the test compound either enhances binding of the calstabin protein to the ryanodine receptor protein, or inhibits dissociation of the calstabin protein from the ryanodine receptor protein.

61. The method of claim 60, wherein the blood sample is a lymphocyte extract.

62. The method of claim 60, wherein the blood sample is from a subject.

63. The method of claim 60, wherein the lymphocyte sample is a blood sample.

64. The method of claim 60, wherein the blood sample is a peripheral blood sample.

65. The method of claim 60, wherein the second blood sample is obtained from the first blood sample, such that only one blood sample needs to be obtained.

66. The method of claim 60, wherein measuring the amount of the calstabin protein bound to the ryanodine receptor protein is performed by immunoprecipating the ryanodine receptor protein complex and detecting, and quantifying the amount of, the calstabin protein present in the immunoprecipated ryanodine receptor protein complex.

67. The method of claim 60, wherein measuring the amount of the calstabin protein bound to the ryanodine receptor protein is performed by immunoprecipating the

calsatbin protein complex and detecting, and quantifying the amount of, the ryanodine receptor protein present in the immunoprecipated calstabin protein complex.

68. The method of claim 66, wherein the ryanodine receptor protein is immunoprecipated using an anti-ryanodine receptor antibody and the calstabin protein is detected using an anti-calstabin antibody.

69. The method of claim 67, wherein the calstabin protein is immunoprecipated using an anti-calstabi antibody and the ryanodine receptor protein is detected using an anti-ryanodine receptor antibody.

70. The method of claim 60, where the ryanodine receptor or the calstabin protein is labeled, and the amount of ryanodine receptor bound to calstabin is determined by detecting and quantifying the label.

71. The method of claim 70, wherein the label is a radiolabel or a fluorescent label.

72. The method of claim 60, wherein the compound is a 1,4, benzothiazepine compound.

73. An *in vivo* method for identifying a compound that produces a change in calstabin binding to a ryanodine receptor, which method comprises
(a) administering to a first animal a physiologically effective amount of a regulating compound that regulates calstabin binding to such receptor;
(b) administering to a second, similar animal the same amount of the regulating compound in combination with an amount of a test compound;
(c) obtaining a sample of blood from the first animal and from said second animal;
(d) measuring calstabin binding to ryanodine receptor in said first and second animal; and
(e) comparing the measured calstabin binding for each animal to determine whether the test compound changes calstabin binding to such ryanodine receptor.

74. An *in vivo* method for identifying a chemical compound that enhances binding of a calstabin protein to a ryanodine receptor protein, or inhibits dissociation of a calstabin protein from a ryanodine receptor protein, comprising:

(a) administering to an animal a physiologically effective amount of a regulating compound that decreases binding of a calstabin protein to a ryanodine receptor protein, or causes dissociation of a calstabin protein from a ryanodine receptor protein;

(b) obtaining a first sample of blood from the animal

(c) subsequently administering to the animal a physiologically effective amount of a regulating compound that decreases binding of a calstabin protein to a ryanodine receptor protein, or causes dissociation of a calstabin protein from a ryanodine receptor protein, and test compound,

(d) obtaining a second sample of blood from the animal,

(e) measuring the amount of a calstabin protein bound to a ryanodine receptor protein in the first blood sample and the second blood sample,

(f) comparing the amount of the calstabin protein bound to the ryanodine receptor protein in the first sample to the amount of the calstabin protein bound to the ryanodine receptor protein in the second sample,

whereby a higher amount of calstabin protein bound to ryanodine receptor protein in the second sample as compared to the first sample indicates that the test compound enhances binding of the calstabin protein to the ryanodine receptor protein, or inhibits dissociation of the calstabin protein from the ryanodine receptor protein.

75. An *in vivo* method for identifying a compound that produces a change in calstabin binding to a ryanodine receptor, which method comprises:

(a) administering to a first animal a physiologically effective amount of a regulating compound that regulates calstabin binding to a ryanodine receptor;

(b) obtaining a first sample of blood from the first animal,

(c) measuring the amount of calstabin bound to the ryanodine receptor in the first blood sample;

(c) administering to a second animal the same relative to weight amount of the regulating compound in combination with an amount of a test compound,

(b) obtaining a second sample of blood from the second animal,

(d) measuring the amount of calstabin bound to the ryanodine receptor in the second blood sample; and

(e) comparing the measured amounts of calstabin bound to the ryanodine receptor in the first and the second blood samples to determine whether the test compound changes the amount of calstabin bound to the ryanodine receptor, wherein an increased amount of calstabin bound to the ryanodine receptor in the second blood sample is indicative of a test compound which increases the binding between calstabin and the ryanodine receptor or inhibits the dissociation of calstabin from the ryanodine receptor.

76. The method of claim 73, 74, or 75, wherein the blood sample is a peripheral blood sample.

77. The method of claim 73, 74, or 75, wherein the blood sample contains lymphocytes.

78. The method of claim 77, wherein the lymphocytes are T cells.

79. The method of claim 77, wherein the lymphocytes are B cells.

80. The method of claim 73 or 75, wherein the first animal and the second animal are substantially genetically identical.

81. The method of claim 73, 74, or 75, wherein the regulating compound is a beta-adrenergic agonist.

82. The method of claim 81, wherein the beta-adrenergic agonist is isoproterenol.

83. The method of claim 73, 74, or 75, wherein the regulating compound is an agonist of the cAMP pathway.

84. The method of claim 73, 74, or 75, wherein the calstabin is calstabin-1, calstabin-2, or a combination thereof.

85. The method of claim 73, 74, or 75, wherein the ryanodine receptor is RyR1, RyR2, RyR3, or a combination thereof.

86. The method of claim 73, 74, or 75, wherein at least one of the compounds is administered subcutaneously.

87. The method of claim 73, 74, or 75, wherein at least one of the compounds is administered via an osmotic pump that is implanted subcutaneously in each of the animals.

88. The method of claim 73, 74, or 75, wherein the regulating compound is administered subcutaneously via an osmotic pump for at least about 3 days.

89. The method of claim 73, 74, or 75, wherein the regulating compound is administered subcutaneously via an osmotic pump for about 5 days.

90. The method of claim 73, 74, or 75, wherein at least one of the compounds is administered subcutaneously or orally

91. The method of claim 73, 74, or 75, wherein the regulating compound is administered subcutaneously via an osmotic and the test compound is administered orally.

92. The method of claim 73, 74, or 75, wherein the test compound is a 1,4, benzothiazepine compound.

93. A method of diagnosing a disease or disorder associated with leak of calcium through a ryanodine receptor in a test subject comprising:
(a) obtaining a sample of blood from a test subject,
(b) obtaining a sample of blood from a control subject known to have normal ryanodine receptor function, and
(c) determining whether there is any calcium leak through the ryanodine receptor in the blood samples obtained in the test subject and the control subject,
whereby a higher level of calcium leak in the ryanodine receptor from the test subject as compared to the control subject, indicates that the test subject may have a disease or disorder associated with the leak of calcium through a ryanodine receptor.

94. The method of claim 93, wherein the blood sample is a peripheral blood sample.

95. The method of claim 93, wherein the blood sample contains lymphocytes.

96. The method of claim 93, wherein the lymphocytes are T cells.

97. The method of claim 93, wherein the lymphocytes are B cells.

98. The method of claim 93, wherein the step of determining whether there is any calcium leak through the ryanodine receptor is performed using an electrophysiological recording technique.

99. The method of claim 93, wherein the step of determining whether there is any calcium leak through the ryanodine receptor is performed using a single channel patch clamp recording.

100. The method of claim 93, wherein the step of determining whether there is any calcium leak through the ryanodine receptor is performed using a fluorescent intracellular calcium indicator.

101. The method of claim 93, wherein the disease and disorder is selected from the group consisting of cardiac disorders and diseases, skeletal muscular disorders and diseases, cognitive disorders and diseases, malignant hyperthermia, diabetes, and sudden infant death syndrome.

102. The method of claim 101, wherein the cardiac disorders and diseases are selected from the group consisting of irregular heartbeat disorders and diseases, exercise-induced irregular heartbeat disorders and diseases, sudden cardiac death, exercise-induced sudden cardiac death, congestive heart failure, chronic obstructive pulmonary disease, and high blood pressure.

103. The method of claim 102, wherein the irregular heartbeat disorders and diseases are selected from the group consisting of atrial and ventricular arrhythmias, atrial and ventricular fibrillation, atrial and ventricular tachyarrhythmias, atrial and ventricular tachycardias, catecholaminergic polymorphic ventricular tachycardia (CPVT), and exercise-induced variants thereof.

104. The method of claim 101, wherein the skeletal muscular disorders and diseases are selected from the group consisting of skeletal muscle fatigue, exercise-induced skeletal muscle fatigue, myopathies, muscular dystrophy, bladder disorders, and incontinence.

105. The method of claim 101, wherein the cognitive disorders and diseases include are selected from the group consisting of Alzheimer's Disease, memory disorders, and age-dependent memory disorders.

106. A method of monitoring ryanodine receptor phosphorylation comprising:

(a) obtaining a first blood sample from a test subject,

(b) obtaining a second blood sample from the test subject, wherein the second sample is obtained after the first sample;

(c) determining the amount of ryanodine receptor phosphorylation in the first sample and the second sample; and

(d) comparing the amount of ryanodine receptor phosphorylation in the first and second sample, whereby a higher amount of ryanodine receptor phosphorylation in the second sample as compared to the first sample indicates progression of a disease or diosrder in the subject.

107. The method of claim 96, wherein the receptor phosphorylation is PKA phosphorylation.

108. A method of monitoring the progression of a disease or disorder associated with abnormal ryanodine receptor comprising:

(a) obtaining a first blood sample from a test subject

(b) obtaining a second blood sample from the test subject, wherein the second sample is obtained after the first sample;

(c) determining the amount of a calstabin protein bound to a ryanodine receptor protein in the first sample and the second sample; and

(d) comparing the amount of the calstabin protein bound to the ryanodine receptor in the first and second sample, whereby a lower amount of calstabin protein bound to the ryanodine receptor in the second sample as compared to the first sample indicates progression of the disease or diosrder in the subject.

109. A method of monitoring drug efficacy in a subject comprising:

(a) obtaining a first blood sample from a test subject who has a disease or disorder associated with abnormal ryanodine receptor;

(b) administering a drug to said subject;

(c) obtaining a second blood sample from the test subject;

(d) determining the amount of a calstabin protein bound to a ryanodine receptor protein in the first sample and the second sample; and

(e) comparing the amount of the calstabin protein bound to the ryanodine receptor in the first and second sample, whereby a higher amount of calstabin protein bound to the ryanodine receptor in the second sample as compared to the first sample is indicative of the efficacy of the drug in increasing the amount of calstabin protein bound to the ryanodine receptor.

110. The method of claim 105, wherein efficacy of the drug in increasing the amount of calstabin protein bound to the ryanodine receptor is indicative of efficacy in treating the disease or disorder associated with abnormal ryanodine receptor.

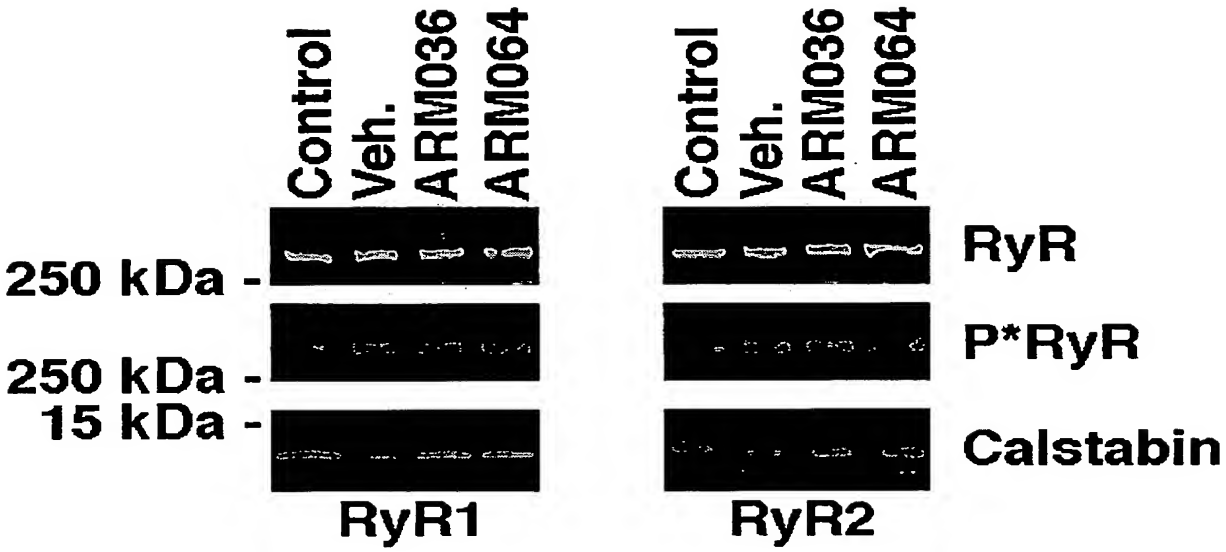
111. The method of claim 106, 108, or 109, wherein the blood sample is a peripheral blood sample.

112. The method of claim 111, wherein the blood sample contains lymphocytes.

113. The method of claim 112, wherein the lymphocytes are T cells.

114. The method of claim 112, wherein the lymphocytes are B cells.

Figure 1


Control
Veh.
ARM036
ARM064
Control
Veh.
ARM036
ARM064
250 kDa -
250 kDa -
15 kDa -
RyR
P*RyR
Calstabin
RyR1
RyR2